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International coordination of research on infectious animal diseases

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 862605

Newsletter

4th Issue August 2022

EDITORIAL

Welcome to the fourth issue of the ICRAD Newsletter!

The Russian military aggression against Ukraine starting in February 2022 has caused a number of problems to ICRAD. Restrictive measures adopted by the European Union and individual member states, involving freezing of funds and assets of Russian entities together with restrictions on scientific collaboration with Russians as such, have urged ICRAD to initiate structural and functional changes of the network. Thus, in order to meet the challenges related to the suspension of ICISTE and MSHE from the Consortium, the tasks of the Russian partners have been taken over by other partners. The positive and flexible approach to facilitate the transfer of these tasks has enabled the activities of ICRAD to continue in accordance with the schedule. At present, the full proposal phase has ended for the Second Call, and the proposals are under evaluation.

Furthermore, the preparation of the Third Call is in good progress. The further process will be announced at a later stage.

The rapidly increasing application of High-Throughput Technologies in veterinary infection biology and diagnostics has provided a unique potential to improve animal health. However, as well-known from the introduction of almost all new promising tools and platforms, lessons from the initial period have to be learnt and addressed to ensure the future successful use.

In this issue of the Newsletter, 3 interesting contributions address some of the advantages and putative challenges related to the practical and scientific use of the High-Throughput Technologies.

Enjoy your reading.

Per Mogensen
ICRAD project manager

Jens Nielsen
ICRAD coordinator

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"Advantages and applications of the High-Throughput Technologies in animal diseases"

Automated high-throughput phenotyping with sensors, imaging, and other on-farm technologies has resulted in a flood of data that are largely under-utilized. Drastic cost reductions in sequencing and other omics technology have also facilitated the ability for deep phenotyping of livestock at the molecular level. These advances have brought the animal sciences to a cross-roads in data science where increased training is needed to manage, record, and analyze data to generate knowledge and advances in Agriscience related disciplines.

" A vision for Development and Utilization of High-Throughput Phenotyping and Big data Analysis in livestock", Coltes et al., 2019, Frontiers in Genetics.

High-throughput screening (HTS) has increasingly been used for novel drug discovery in the field of pharmaceutics replacing the traditional "trial and error" approach to identify therapeutic targets and validate biological effects. HTS involves assaying and screening a large number of biological effectors and modulators against designated and exclusive targets. Thus, HTS is generally favored when little is known of the target, which precludes structure-based drug design, but it can also be used in parallel with other strategies such as computational techniques and fragment-based drug design.

"High-Throughput Screening Platforms in the Discovery of Novel Drugs for Neurodegenerative Diseases", Aldewachi et al., 2021, Bioengineering.

As demonstrated by several peer-reviewed articles, HTS has shown great potential in the detection and discovery of novel pathogens (see 'Examples of the application of high-throughput sequencing in veterinary diagnostic microbiology', below). In this regard, it is common to distinguish between the spread of known infections to new areas and/or the emergence of completely novel, 'unknown' pathogens. Contrary to earlier techniques, HTS is unbiased and reports all nucleotide sequences present in the original sample. However, as with earlier techniques, the lower limit for detection is still ultimately determined by the abundance of pathogens in relation to host background material. By enabling deeper sequencing to be performed more quickly and cheaply, the continuing development of HTS techniques is also continuing to improve the likelihood of detecting low copy-number pathogens.

"High-throughput sequencing in veterinary infection biology and diagnostics", Belak et al., 2013, Rev. sci. tech. Off. int. Epiz.

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Special focus on-

BIOSENS4PRECISIONMASTITIS

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Biosens4PrecisionMastitis aims to deliver new diagnostic tools to detect mastitis at the early stages of the disease, by targeting biomarkers of the early immune response of cows: miRNAs, cytokines and antimicrobial peptides. The project was set to work with naturally infected cows to initially identify key bovine mastitis biomarkers in milk. Researchers at the Research and Innovation Centre Pro-Akademia (RIC) collected representative samples from the herd of ~35 Holstein-Friesian dairy cows in two rounds (spring and autumn 2021). After identifying based on somatic cell count (SCC) values five healthy cows, five cows with clinical mastitis and five cows with subclinical mastitis, milk from those selected animals was collected on three consecutive days. Added to SCC, samples were analysed by microbial culturing to determine the total number of bacteria, and to detect the most common microorganisms causing mastitis, including S. agalactiae, S. uberis, S. aureus, E. coli, etc. Microbiological results did not reflect infection's severity but can be useful to guide the antibiotic therapy of cows with mastitis at an advanced stage.

Researchers at RIC isolated total RNA from milk samples to be used at SeqOmics for high-throughput NGS-based miRNA analysis with the aim of selecting miRNA sequences key at the early stages of bovine mastitis. The total isolated RNA (from 45 samples) was quality-checked using an Agilent Tapestation system. Equimolar amounts of pooled total RNA samples were used for miRNA in vitro fragment library preparation. The libraries were sequenced on an Illumina NextSeq1000 platform. 1-2 millions of paired end reads (2x 150 nt each) were generated for each one of the three pools prepared using healthy, clinical and subclinical mastitic cows' samples, which analysis provided unique miRNA patterns. Specific miRNA species showing increased expression level in the clinical and subclinical samples were selected as potential biomarker candidates, some of them agreeing with previously identified miRNAs for bovine mastitis (e.g. miRNA221, miRNA223, miRNA146b, miRNA29a, miRNA29c). Validation of the selected miRNA species has begun using qRT-PCR approach.

In addition, RIC's team isolated milk sediments to be used by researchers at the Riga Stradiņš University (RSU) to analyse the occurrence of cytokines and antimicrobial peptides produced in the host organism as early biomarkers of mastitis. Milk smears of 30 cows (10 healthy, 10 with subclinical mastitis, 10 with clinical mastitis) were prepared and immunohistochemically stained with 16 factors (Hdef2, Hdef 3, IL-10, IFNg, IL-2, LL-37, IL-1a, IL-4, IL-6, IL-12, IL-13, IL-17A, TNFα, TGFβ1, NFκB, IL-8) at six different days. A total of 1,200 preparations were then analysed. From data corresponding to samples collected in spring 2021 (15 cows data), it was observed that in healthy cows, high numbers of immunoreactive cells indicated readiness of host immunity under continuous pathogen exposure. Consequently, it was confirmed that both local cellular and humoral immune responses play a role in defense responses. Data from mastitic cows showed that inflammatory responses to intramammary infection were driven by IL-1α, IL-4, IL-12, IL-17A, IFN-γ in subclinical mastitis, and by IL-1α, IL-4, IL-6, and IL-17A in clinical mastitis. From the whole collected data (30 cows), researchers at RSU identified two factors as potential biomarkers for the prompt detection of bovine mastitis: IL-10 which provides anti-inflammatory defence, and β-defensin 3 with microbicidal activity towards Gram-negative bacteria, yeasts, and some Gram-positive bacteria. Interestingly, there are four other factors, IL-2, IL-4, TGFβ1, and Il-17A, which marked positively almost 90-100% of milk smear cells in both healthy and diseased animals.

At the University Rovira i Virgili (URV) researchers worked on developing a novel sensing platform able to provide the highest sensitivity, selectivity and accuracy for the detection of the key biomarkers previously identified. First, a new porous silicon (pSi) nanostructured electrochemical transducer prepared by polymerisation and carbonisation of furfuryl alcohol was fabricated and characterised, showing outstanding electrochemical performance with fast electron transfer kinetics, large effective surface area with low background current, wide potential window, and high reproducibility and stability. To demonstrate its biosensing capabilities towards the detection of miRNAs, the platform was initially tested against a model target ssDNA, underpinning the feasibility to detect low pM concentrations of the analyte, as shown in Figure 1. Current work focuses on optimising the biosensig platform (i.e. pore size, capture probe concentration, incubation time), and adapting its morphology and chemical modification to the detection of IL-10.

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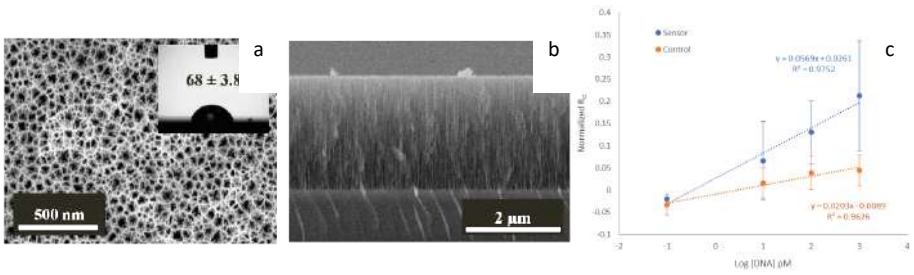

Figure 1: (a) Top and (b) cross-section FESEM images of a pSi electrode carbon-stabilised via furfuryl alcohol polymerisation followed by carbonisation (PFApSi). (c) Dose-response curve for the detection of ssDNA (as model for miRNA) obtained using a DNA-modified PFApSi electrode. Electrochemical impedance spectroscopy measurements were recorded in a 2 mM [Fe(CN)6]3-/4- solution.

Our first research results have been presented at the 2nd Natural Scientific Conference InnWet in Lublin (Poland), the 7th International Conference on Bio Sensing Technology in Sitges (Spain), the International Nanotech & Nanoscience Conference & Exhibition, Nanotech France 2022 in Paris (France), and Transfiere, 11th European Meeting on Science, Technology and Innovation in Málaga (Spain). Results have been disseminated through publications in Medicus Bonus. A paper entitled "Identification of Inflammatory and Regulatory Cytokines IL-1α-, IL-4-, IL-6-, IL-12-, IL-13-, IL-17A-, TNF-α-, and IFN-γ-Producing Cells in the Milk of Dairy Cows with Subclinical and Clinical Mastitis" has been published in the journal Pathogens (doi.org/10.3390/pathogens11030372).

THE ROLE OF HIGH-THROUGHPUT ANALYSIS TECHNIQUES IN PRECISION LIVESTOCK FARMING

Beatriz Prieto-Simón, ICREA Research Professor at the University Rovira i Virgili, Spain

To improve performance and well-being of livestock, animal health management has experienced a shift from a reactive approach, focused on treating animals to cure them, to a pro-active attitude where prevention is paramount. In the specific case of animal infectious diseases, diagnostic tools enabling prevention of disease progression and spread to other animals can have major impact. The advantages of adopting a preventive health management are not only limited by the benefits to individual animals, but also by the support they can provide to identify and isolate the source of the infection outbreak, and thus improve infection control. The adoption of improved infection control practices contributes to reducing the overuse and misuse of antimicrobials, underpinning a paradigm shift in antimicrobial stewardship interventions addressed to reduce the emergence of antimicrobial resistance [1].

To prevent animal diseases, it is of utmost importance to design workflows that can be easily adopted by farmers to monitor the overall health status of animals. These workflows must be supported by new tools able to record physiological and environmental conditions, and/or molecular information.

On the one hand, sensors used to assist farmers in adapting their practices to animals' physiological status and environmental conditions provide data which can be used to identify clinical signs of animals' diseases. Wireless sensors attached to animals (e.g. smart neck-mounted collars, ear tags, etc) have been used to monitor location, feeding habits, etc, providing real-time data about herd's health [2]. The development of new reliable, cost-effective, low-power network technologies and smart sensors is leading to a new era of precision livestock farming based on data-driven decision-making. The data acquired can be harnessed to identify early signs of emerging health issues on an individual animal basis allowing farmers to pro-actively take measures to confirm disease aetiology and guide therapy.

On the other hand, molecular-based diagnostics have revolutionised the way veterinarians screen, diagnose and monitor diseased animals. Molecular diagnostics include genetic tests, mostly based on DNA sequencing, and tests targeting key biomarkers of disease (e.g. miRNAs, proteins, metabolites, etc) [3]. The development of these tests has raised in parallel to intense research efforts devoted to identifying new biomarkers [4–7]. Added to biomarkers confirming a specific disease, biomarkers associated to the host-immune response are key to diagnose the onset of a disease, even before symptoms appear what is expected to become a breakthrough in animal health management [8]. To maximise the potential of molecular diagnostics to support animal disease detection, management, control and eradication, the newly developed tools must surpass conventional analysis techniques in terms of high-throughput and cost-efficiency. On-farm high-throughput molecular diagnostics can be envisaged both as screening tools, requiring confirmation in the laboratory by using standard methods of analysis, and as powerful tools for accurate diagnosis. The latter have to excel in terms of sensitivity and specificity.

Advances in high-throughput DNA sequencing platforms have been essential to identifying genes associated to specific pathogens causing animal infections, disease-related mutations of animal genes, and more recently changes in gene expression caused by the animals' immune response to a specific pathology. The genetic knowledge acquired is currently harnessed by rapid nucleic acid-based diagnostics, especially those relying on polymerase chain reaction (PCR) amplification, widely applied in animal diagnostics [9]. The development of portable thermocyclers and the use of lyophilised reagents, have eased the adoption of real-time PCR in the field, becoming a powerful rapid decision-making tool. Nevertheless, this technique suffers from both false positive and negative results. For screening purposes, false positives, mainly caused by contaminations prone when tests are performed outside laboratory environments, can be accepted, as results need to be verified in the laboratory. However, for accurate diagnosis, false negative results are not acceptable. Moreover, the analysis time of real-time PCR is still too long to suit on-farm monitoring of animals.

Indeed, to facilitate on-farm adoption, diagnostic tools have to be simple, fast and low cost. Rapid diagnostic tests (RDTs) are easy-to-use tools that suit on-farm measurement conditions, as they do not require trained personnel and deliver results on-site in less than 20 minutes. Among RDTs, lateral flow immunochromatographic tests have been developed for a wide range of animal diseases mostly targeting nucleic acids, metabolites,

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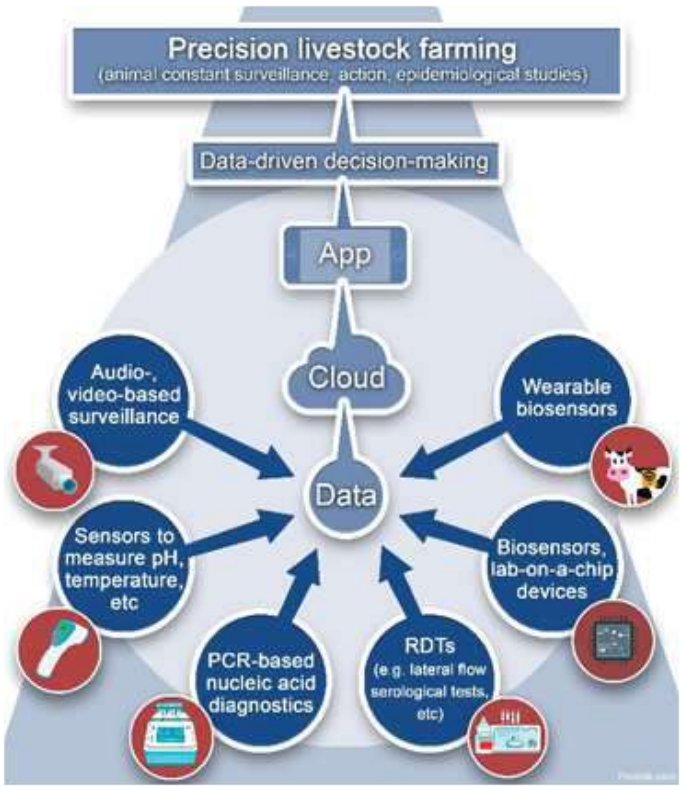
antigens or antibodies, demonstrating their potential as on-farm tools [10]. Lateral flow devices include all steps from sample-to-result, but due to their simplicity they are limited in terms of the sensitivity they can reach, or the type of biofluid (e.g. milk, saliva, urine, blood) they can deal with.

In this context, key advances in biosensing technology have been key to develop highly sensitive, selective, simple, cost-effective and accurate diagnostic tools able to detect key biomarkers directly in complex samples. Thanks to the versatility in their design, allowing the selection of the most appropriate transducer, bioreceptor, sensing mechanism and detection technique, biosensors are often developed as powerful fit-for-purpose alternatives to laboratory-based diagnostic techniques [11]. Moreover, their miniaturisation and integration within microfluidics have sparked the development of portable laboratories, able to perform measurements on-site, with minimal hands-on intervention from sample introduction to the delivery of results [12]. The resulting lab-on-a-chip devices allow automation to facilitate continuous monitoring of animals on-farm, and thus are suitable to be integrated within high-throughput workflows. Nevertheless, the intensive research efforts devoted to the development of biosensors have only occasionally led to technology adoption. There are several reasons for that, but most likely the limitations in technology translation come from their often-poor reproducibility and reliability, lack of demonstration with real samples due to limitations caused by matrix effects, and eventually, their limited technological maturity. Among the various strategies explored to tackle the setbacks that have slowed down for years the translation of the scientific and technological knowledge acquired in biosensors into cost-effective, and highly efficient diagnostic devices to support animals' health management, the design of new innovative sensing principles, and the incorporation of highly sensitive transducers, and specific and robust bioreceptors are paving the way towards the next generation of animal diagnostics.

Such biosensing technology in animal diagnostics has been taken to a next level by the growing interest in wearable technologies [13]. Similar to the already well-established wearable biosensors supporting humans' health and wealth, the advent of a new class of non-invasive or minimally invasive biosensing devices attached on animals to monitor their health status through an automated and high-throughput workflow, is blooming. Interestingly, the level of digitalisation already achieved thanks to the advancements on the Internet of Things sensor technology devoted to collecting physiological and environmental data, is now applied to these novel wearable biosensors. The possibility to continuously collect data on key biomarkers for all animals in a herd, and send such information online to a centralised platform for its analysis, favours the quick response to cases that require action. Furthermore, the amount of data generated by such high-throughput techniques is key to support animal surveillance strategies and epidemiological studies.

Covid19 pandemics has evinced the impact that diagnostics easily and quickly deployable on site can have on the identification and control of a health threat. The lessons learnt and the awareness of the huge impact that improper diagnosis and treatment of animal diseases can have on animals' health and welfare, livestock performance, the environment, the economy, and in general on our society, underpin the need to identify high-throughput analysis techniques helping to prevent animal diseases. Furthermore, these techniques must also contribute to increase our preparedness to deal with existing and emerging animal diseases. In this context, biosensing devices that can be integrated within existing farm infrastructures or attached on animals as wearable devices, able to non-invasively or minimally invasively monitor in close-to-real-time key biomarkers, especially those related to the early host-immune response, are emerging as the cornerstone of precision livestock farming.



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ADVANTAGES AND APPLICATIONS OF HIGH-THROUGHPUT ANALYSIS TECHNIQUES IN PRECISION LIVESTOCK FARMING

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Accurate diagnosis and safe identification of several animal diseases is a big challenge nowadays. Despite serology has been used on a wide scale in diagnosis, still, isolation and identification of the causative pathogen are the gold standards for diagnosis either by classical bacteriology or molecular diagnosis based on several PCR protocols. Over the last decade, several outbreaks of animal diseases have been reported worldwide, however, data on the genetic diversity and the ability to trace back the infection are usually incomplete. Classical identification and diagnostic tools are able to define the pathogen, but these tools provide no in-depth characterization regarding genetic diversity, the lineage of isolates, and the source of infection. Whole-genome sequencing (WGS) has rapidly become a valuable method in clinical microbiology laboratories. It allows the characterization of bacterial pathogens simultaneously at multiple levels, including species determination, genotype, prediction of resistance and virulence factors, and analysis of population structures. The use of whole genomic sequencing (WGS) for monitoring certain bacterial pathogens may even become mandatory in the near future, as it has been recommended for example by the European Food Safety Authority. WGS is a sensitive, and time-saving alternative to the classical methodology that enables high-resolution characterization of isolates at the strain or clone level. This high-resolution genotyping is valuable from a "One Health" perspective to detect outbreak clusters, identify sources of infection, and track pathogen transmission and spread. However, it requires meticulous harmonization to compare results across sectors and transdisciplinary. WGS has the advantage to offer several options for genotyping. This includes the detection of sequence variants, especially single nucleotide polymorphisms (SNP), by mapping 'raw sequences' obtained from an isolate to a reference genome, aligning assembled core genomes, or applying kmer-based analysis, where the resulting SNP matrix can be used for informative phylogenetic analyses. Other genotyping methods rely on the detection of gene variants in the form of numbered allele profiles, e.g., in multilocus sequence typing, which in principle converts unique gene sequences into unique numbers to facilitate data portability and communication.

Conventional MLST uses only a few genes for typing, making it difficult to distinguish between outbreak strains, especially in monomorphic species such as Bacillus anthracis and Brucella species. Genomic MLST, on the other hand, is an extension of this traditional approach where all 'typeable' genes can be considered, thus improving resolution. MLST data are 'lightweight' and thus can be easily shared via online nomenclature databases, in addition to that the underlying analytical workflow is less computationally intensive. Therefore, parallel analysis based on sequence variants is recommended. Sequence data of low quality can be removed from the original alignment file, resulting in data of high quality for downstream analysis. In addition, phylogenetic reconstruction of SNP alignment data is robust because it is based on base-by-base comparison and considers evolutionary models with masked recombination sites and mobile elements. It should be reiterated that processing SNP alignment data can be computationally intensive and requires prior bioinformatics knowledge.

On the other hand, the burden of antimicrobial resistance (AMR) is on the rise globally, thus, investigation of the virulence-associated genes and antimicrobial resistance determinants is a demand, particularly in highly pathogenic bacteria such as biosafety level group agents. Although several PCR protocols have been designed to identify the virulence-associated genes, these tools are limited and identify only the expected genes based on the chosen primers. In the era of NGS, where a huge amount of data can be retrieved, the implementation of high-throughput WGS for isolates can facilitate rapid and comprehensive detection and distribution of virulence and AMR genes.

In summary, the current options for genotyping bacterial strains using WGS have different advantages. A combined application of the methods allows accurate tracing of entry sources and identification of transmission pathways, but also global molecular surveillance becomes possible. Various typing methods have been developed for animal pathogens now available internationally.

an update on the Second Call

Second call is focused on ***"One Health Approach to Zoonoses Research and Innovation"***.

The overall goal of this ICRAD call is to support cross-cutting research and innovation to better understand zoonoses focusing on the animal-human-environment interface and by developing novel vaccine and diagnostics technology platforms to improve animal health and by consequence animal welfare.

The objective of this funding call is to increase preparedness and improve the ability to respond to (re)-emerging zoonotic disease threats and contribute to improved animal and public health. This will be done through studies focusing on (re)-emergence of pathogens with zoonotic potential, understanding animal host-pathogen interactions and the immune response, and by developing detection and prevention platforms. Proposals should include research on one of the following topics:

Research area 1: Improved understanding of animal-human-environment interface

- Pathogen (Re)-Emergence and Host Adaptation
- Host/Pathogen Interactions

Research area 2: Detection and Prevention

- Vaccine Technology Platforms
- Diagnostic Technology Platforms

The full proposal phase has ended and 24 full proposals submitted in time. The submission tool is closed and next step is the general eligibility check and after that the national eligibility check.

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